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Amyloid beta selectively modulates neuronal TrkB alternative transcript expression with implications for Alzheimer's disease

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Abstract

Dysregulation in brain-derived neurotrophic factor (BDNF)/full-length TrkB (TrkB-TK+) signaling is implicated in promoting neurodegeneration in Alzheimer's disease (AD). BDNF/TrkB-TK+ signaling can be modulated by the presence of truncated TrkB isoforms (TrkB-TK-, TrkB-Shc). All TrkB isoforms are encoded by different alternative transcripts. In this study, we assessed if expression of the three main TrkB alternative transcripts, TrkB-TK+, TrkB-TK-, and TrkB-Shc are altered in AD. Using a cohort of control and AD brains (n=29), we surveyed the hippocampus, temporal cortex, occipital cortex, and cerebellum and found specific increases in TrkB-Shc, a neuron-specific transcript, in the AD hippocampus. No significant changes were detected in TrkB-TK+ and TrkB-TK- transcript levels in AD in any brain region examined. Corresponding changes in truncated TrkB protein levels were found in the hippocampus, although a significant increase in the temporal cortex was also observed. Our findings suggested that neuron-specific changes in TrkB may be occurring in AD; thus, we determined whether TrkB-Shc expression could be modulated by amyloid beta 1-42 (Aβ 42). We found increased TrkB-Shc mRNA levels in differentiated SHSY5Y neuronal cell-lines exposed to fibril-containing Aβ 42 preparations. When we assessed the cellular impact of increased TrkB-Shc, we found co-localization between TrkB-Shc and TrkB-TK+. Interestingly, TrkB-Shc overexpression selectively attenuated BDNF/TrkB-TK+-mediated signaling via the mitogen-activated protein kinase kinase (MEK) pathway, but not the protein kinase B pathway. In AD, MEK signaling is increased in vulnerable neurons and linked to abnormal phosphorylation of cytoskeletal proteins. Altogether, our findings suggest that elevated TrkB-Shc expression in AD may function as a compensatory response in neurons in AD to promote survival.

Keywords

expression, alzheimer, transcript, alternative, trkb, neuronal, modulates, selectively, beta, amyloid, implications, disease

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Amyloid beta selectively modulates neuronal TrkB alternative transcript expression with implications for Alzheimer's disease

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Abbreviations

AD, Alzheimer's disease; ATRA, all-trans retinoic acid; Aβ amyloid beta; BDNF, brain derived neurotrophic factor; TrkB, tropomyosin receptor kinase B; PMI, postmortem interval; qPCR, quantitative real-time PCR

Abstract

Dysregulation in brain-derived neurotrophic factor (BDNF)/full-lengthTrkB (TrkB-TK+) signaling is implicated in promoting neurodegeneration in Alzheimer's Disease (AD). BDNF/TrkB-TK+ signaling can be modulated by the presence of truncated TrkB isoforms (TrkB-TK-, TrkB-Shc). All TrkB isoforms are encoded by different alternative transcripts. In this study, we assessed if expression of the three main TrkB alternative transcripts, TrkB-TK+, TrkB-TK-, and TrkB-Shc are altered in AD. Using a cohort of control and AD brains (n=29), we surveyed the hippocampus, temporal cortex, occipital cortex, and cerebellum and found specific increases in TrkB-Shc, a neuron-specific transcript, in the AD hippocampus. No significant changes were detected in TrkB-TK+ and TrkB-TK- transcript levels in AD in any brain region examined. Corresponding changes in truncated TrkB protein levels were found in the hippocampus although a significant increase in the temporal cortex was also observed. Our findings suggested that neuron-specific changes in TrkB may be occurring in AD, thus we determined whether TrkB-Shc expression could be modulated by amyloid beta 1-42 ($A\beta_{42}$). We found increased TrkB-Shc mRNA levels in differentiated SHSY5Y neuronal cell-lines exposed to fibril-containing $A\beta_{42}$ preparations. When we assessed the cellular impact of increased TrkB-Shc, we found co-localization between TrkB-Shc and TrkB-TK+. Interestingly, TrkB-Shc overexpression selectively attenuated BDNF/TrkB-TK+-mediated signaling via the MEK pathway but not the AKT pathway. In AD, MEK signaling is increased in vulnerable neurons and linked to abnormal phosphorylation of cytoskeletal proteins. Altogether, our findings suggest that elevated TrkB-Shc expression in AD may function as a compensatory response in neurons in AD to promote survival.

Key Words: TrkB, BDNF, amyloid, neurotrophin, neurodegeneration, hippocampus

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases in humans and is characterized by amyloid-β (Aβ) deposition, tau neurofibrillary tangles, and neuronal loss. Deficits in brain-derived neurotrophic factor (BDNF) signaling have been proposed to facilitate the progressive neurodegeneration in AD (Schindowski et al., 2008). The growth promoting actions of BDNF are critical for survival and plasticity of a variety of neurons throughout the brain. Moreover, BDNF has been demonstrated to be vital for neuronal growth, differentiation, axonal guidance, behavior, cognition and memory formation; and hippocampal neurogenesis (Knusel et al., 1991, Ghosh et al., 1994, McAllister et al., 1995, Itami et al., 2000, Mizuno et al., 2000, Alonso et al., 2002, Lu et al., 2008, Schindowski et al., 2008, Gao and Chen, 2009, Gao et al., 2009). The neurotrophic effects of BDNF are mediated by the tropomyosin receptor kinase B (TrkB), a membrane-bound receptor tyrosine kinase which can activate various cell signaling pathways linked to growth, differentiation, and survival (Patapoutian and Reichardt, 2001, Bartkowska et al., 2010).

The TrkB gene is expressed as multiple alternative splice transcripts that give rise to different protein isoforms (Luberg et al., 2010). Three major TrkB transcripts/isoforms are expressed in the human brain (Stoilov et al., 2002, Luberg et al., 2010). The high affinity, full-length (TrkB-TK+) TrkB receptor is encoded by 24 exons and is expressed primarily by neurons. It contains a catalytic tyrosine kinase domain that is necessary for activating second messenger signaling via the MEK, AKT, and PLCγ pathways to mediate the neurotrophic effects of BDNF (Klein et al., 1991, Stoilov et al., 2002, Luberg et al., 2010) (Fig. 1). The C-terminal truncated TrkB receptors include TrkB-TK- and TrkB-Shc. TrkB-TK- is encoded by 16 exons and is expressed primarily by glia, although expression in neurons has also been reported (Stoilov et al., 2002, Ohira and Hayashi, 2003, Ohira et al., 2005a, Ohira et al., 2005b, Luberg et al., 2010). Unlike TrkB-TK+ transcripts which do not have exon 16, TrkB-TK- transcripts include exon 16. Exon 16 encodes a stop codon and this leads to the

generation of a short isoform-specific C-terminal domain that lacks the catalytic tyrosine kinase domain (Klein et al., 1990) (Fig. 1). Thus, TrkB-TK- may inhibit neurotrophin signaling by sequestering or trapping neurotrophins to prevent binding and signal transduction via TrkB-TK+ homodimers (Biffo et al., 1995, Fryer et al., 1997), or act in a manner similar to "antigen presenting" receptors by binding to BDNF and presenting it to nearby cells expressing TrkB-TK+ (Beck et al., 1993, Frisen et al., 1993). Alternatively, TrkB-TK- has been demonstrated in vitro to act as a dominant-negative receptor when co-expressed in the same cell type by forming inactive heterodimers with TrkB-TK+ to prevent neurotrophin signaling (Eide et al., 1996, Ninkina et al., 1996). The other major C-terminal truncated TrkB receptor isoform, TrkB-Shc, differs from TrkB-TK- in that it is expressed only by neurons (Stoilov et al., 2002). It contains a sarc homology binding site (Shc) in the juxtamembrane domain similar to TrkB-TK+, however, TrkB-Shc transcripts include exon 19 which is not present in TrkB-TK+ transcripts. Exon 19 also contains a stop codon that leads to the translation of a unique truncated C-terminus which also lacks the tyrosine kinase domain (Stoilov et al., 2002) (Fig. 1). Thus, TrkB-Shc may also act as a negative regulator of TrkB-TK+ as it is able to dimerize with TrkB-TK+ but is unable to signal via tyrosine kinase (Stoilov et al., 2002).

At present, studies examining changes in TrkB mRNA and protein expression in AD are incomplete and conflicting changes in TrkB-TK+ and TrkB-TK- expression have been reported. In AD brains, reductions in TrkB-TK+ protein in neurons have mostly been found in the hippocampus and the temporal and frontal cortices (Allen et al., 1999, Ferrer et al., 1999). Others have reported no loss of TrkB immunnoreactivity in AD parietal cortex, however this was using an antibody to TrkB-TK- (Savaskan et al., 2000). Moreover, previous studies of TrkB expression in the hippocampus of AD brains have only examined TrkB-TK+

and TrkB-TK- protein expression by immunohistochemistry and western blotting (Connor et al., 1996, Ferrer et al., 1999).

Interestingly, while the existence of TrkB-Shc has been known for some time, its role in AD has yet to be described. Considering that TrkB-Shc has been characterized to be expressed only in neurons (Stoilov et al., 2002), and that neurotrophin signaling is decreased in neurons in AD, it is important to establish what role TrkB-Shc plays in AD development and progression.

In the present study, we took a quantitative approach to specifically measure the expression levels of each of the three major TrkB alternative splice variants (TrkB-TK+, TrkB-TK-, and TrkB-Shc) in the hippocampus (a region heavily impacted in AD), temporal cortex (a region also affected in AD), occipital cortex (an area affected late in AD), and cerebellum (a region relatively spared in AD) of AD brains. Considering that TrkB-Shc is neuronal specific, we also determined if changes in its expression could be modulated in an amyloidogenic environment and whether increased TrkB-Shc has a functional impact on BDNF/TrkB-TK+ signaling in a neuronal setting.

Experimental Procedures

Brain cohorts

Two brain cohorts were used in this study. For the hippocampus and cerebellum, the brain cohort comprised n=6 controls and n=6 AD cases (Sydney Brain Bank and New South Wales Tissue Resource Centre, Sydney, Australia). For the temporal and occipital cortices, the brain cohort comprised n=8 controls and n=9 AD cases (this was generously provided by Professor Glenda Halliday). All cases were longitudinally evaluated to autopsy, and data and brain collected for research purposes as approved by Institutional Human Ethics Committees. AD brains were clinically and pathologically defined using NIA-Reagan criteria as described previously (NIA, 1997, Gregory et al., 2006, Kim et al., 2010). All AD cases had Braak neuritic stages V and VI. Demographic data for the cohorts are detailed in Table 1. Control and AD cases were matched for age and gender where possible. The diagnostic groups did not differ according to age, brain pH, or postmortem interval (PMI) (all $p \geq 0.12$). Samples were taken from the same anatomical regions of the brain, as previously described (Kim et al., 2010)

Cell culture and treatments

SHSY5Y cells were obtained from the American Type Culture Collection (ATCC) and grown at 37°C in a 5% CO_2 atmosphere. For continuous culture, SHSY5Y cells were cultured in RPMI media containing 10% (v/v) FBS supplemented with glutamax (2 mM). For experiments, SHSY5Y cells were differentiated for 9 days using all-trans retinoic acid (ATRA:10 μM) which was added to the culture medium. During differentiation, the differentiation media (growth media + ATRA) was refreshed every 3 days. For transfections, differentiated SHSY5Y cells were transfected using OptiMEM medium and Lipofectamine

2000 (Invitrogen). For treatments, serum-free RPMI media supplemented with N2 was used. Cells were treated with BDNF for 15 min prior to harvest. For $A\beta_{42}$ experiments, cells were treated for 6 h and harvested. No cell-toxicity was observed in any of the treatment conditions at the concentration employed.

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR)

Total RNA was extracted from tissues (20 mg) and cells using Trizol Reagent according to the manufacturer's instructions (Invitrogen). Concentrations of total RNA were measured using a Nanodrop 2000c Spectrophotometer (ThermoScientific). Reverse Transcriptase-PCR was performed according to the manufacturer's protocol for the SuperScript III First Strand cDNA Synthesis Kit (Invitrogen). QPCR was performed using Sso Fast Evagreen Supermix (BioRad) on a Roche LightCycler 480 with a 96-well format (Roche). Primer pairs used for gene expression analyses are listed in Table 2. Primers were designed to span an intron/exon boundary to avoid amplification of possible genomic DNA. PCR cycling conditions were: 95°C for 1 min, 45 cycles of 95°C for 30 seconds, primer specific annealing temperature for 30 seconds (Table 2), and 72°C for 30 seconds. Control reactions with no template were included which produced no signal. Melt curve analysis and agarose gel electrophoresis were performed to confirm production of a single product. Changes in gene expression levels were determined by normalizing mRNA levels of the gene of interest to the mRNA level of the housekeeping gene, β-actin using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). β-Actin did not vary with treatment and there was no difference in β-actin expression between control and AD cases in any of the brain regions examined (all $p>0.06$) (Fig. S1A). For details on housekeeping gene selection, please refer to the Supplemental Results.

$A\beta_{42}$ preparation

$A\beta_{42}$ monomers, oligomers, and fibrils were prepared using a modified protocol from Ryan et al. (Ryan et al., 2010). Lyophilized $A\beta_{42}$ peptides were reconstituted in DMSO to a working concentration of 1 mM. For experiments, 1 μM was used as the final concentration. The volume required for each experiment was aliquoted for preparation. For monomers, aliquots were sonicated for 10 min and briefly vortexed. For oligomer preparations, after sonication, monomers were diluted 1:10 using PBS+0.05% SDS, vortexed for 30 seconds, and incubated at 4°C for 40 h. For fibril preparations, after sonication, monomers were diluted 1:10 using PBS, vortexed for 30 seconds, and incubated at 37°C for 40 h.

Atomic Force Microscopy (AFM)

$A\beta_{42}$ samples (20 μl) prepared as described above were pipetted onto the surface of freshly cleaved 1 x 1 cm mica. Samples were incubated on the mica surface for 30 min to allow for sufficient adsorption of the $A\beta_{42}$ samples and then washed by exchanging the sample solution (six times) with nanopure water. The $A\beta_{42}$-coated mica surface was then air dried and mounted on the AFM sample stage and static mode force measurements were performed using an Asylum Research MFP-3D AFM (Santa Barbara, CA) as previously described (Higgins et al., 2006). Images were captured using the Asylum Research AFM IGOR Pro software (Wavemetrics, Lake Oswego, OR).

Immunofluorescence staining and fluorescence microscopy

SHSY5Y cells were seeded in 24-well plates containing sterile 12 × 12 mm glass coverslips (density of: 6×104) and differentiated for 9 days as described above. Cells were transfected with overexpression plasmids [pcDNA3.1-TrkB-Shc-myc and pcDNA3.1-TrkB-TK+-EGFP (constructed and validated by GeneArt)] (100 ng/well) for 24 h using Lipofectamine 2000 (1 μl/well) and washed twice with PBS and fixed with 3% (v/v) formaldehyde/PBS for 15 min

at room temperature. After washing with PBS (3×5 min), cells were permeabilised with 0.1% (v/v) Triton X-100 in PBS (5 min). After washing with PBS (3×5 min), cells were incubated with 10% (v/v) FBS in PBS for 1 h at room temperature. Cells on the coverslips were then incubated with primary antibody against myc (Invitrogen) 2 μg diluted in 10% (v/v) FBS in PBS plus 0.1% (w/v) saponin for 16 h at 4 °C. After washing with 10% (v/v) FBS in PBS (3×5 min), cells were incubated with 5 μg/ml of Alexa Fluor-594 conjugated mouse secondary antibody (Molecular Probes) for 1 h at room temperature. Cells were washed with 10% (v/v) FBS in PBS (3×10 min). Coverslips were then mounted on glass slides using ProLong® Gold antifade reagent with DAPI (Molecular probes). Images were obtained using a Nikon ECLIPSE RE2000-E micorscope (Nikon, Japan).

Western blotting

Tissues (10mg) and cells were harvested for total protein using homogenization buffer (human tissue) (Wong *et al.*, 2009) or RIPA buffer (cells) supplemented with protease inhibitors (2 mM AEBSF, 0.015 mM aprotinin, 0.038 mM leupeptin, 0.030 mM pepstatin A, 0.028 mM E-64, 0.08 mM bestatin) (Sigma) and phosphatase inhibitors (Pierce). Protein concentrations were determined by the bicinchoninic acid method. Samples (including $A\beta_{42}$ preparations) were mixed with 5 × SDS loading buffer (containing β-mercaptoethanol), boiled at 95ºC for 5 min, and separated on 10% or 13% SDS-PAGE gels (13% was used for $A\beta_{42}$ preparations). Proteins were transferred onto nitrocellulose membranes and blocked using 5% (w/v) non-fat milk, 0.1% (v/v) Tween-20 in TBS (TBST) at room temperature for 1 h. For membranes containing $A\beta_{42}$ samples, membranes were boiled in PBS for 5 min prior to blocking to expose epitopes in oligomers and fibrils (Takahashi et al., 2004). Membranes were incubated with primary antibodies: WO2 (1:500 dilution), which recognizes the Aβ peptide; TrkB (1:3000) (BD Biosciences); phospho-TrkB (1:5000) (Epitomics); AKT1

(1:5000), phospho-AKT1 (1:5000), ERK1/2 (1:20000), phospho-ERK1/2 (1:20000) (Cell Signaling), myc (1:5000) (Invitrogen), and β-actin (1:10000) (Sigma). Membranes were washed 3 x 10 min with TBST and incubated with mouse peroxidase-conjugated affinity purified secondary antibody for 1 h (Dako). After further washing, bound antibodies were incubated with enhanced chemiluminescence reagent (Millipore) and visualized by autoradiography. Bands were quantitated by densitometry using Image J (version 1.37v) (National Institutes of Health USA). The brightness/contrast of images was adjusted using Adobe Photoshop CS (version 8).

Statistical analysis

All cell culture experiments were from 3 replicate cultures representative of 3-6 independent experiments. Data are presented as mean + standard error of the mean (SEM). Outliers were determined and removed from subsequent analyses using the Grubb's test or if data points were greater or less than two standard deviations from the mean (qPCR: Hippocampus: TrkB-Shc: one control and one AD case removed; TrkB-TK-: one control and one AD case removed; TrkB-TK+: one control removed; Temporal cortex: TrkB-Shc: one AD case removed; TrkB-TK-: one control removed; TrkB-TK+: one control removed; Occipital cortex: TrkB-Shc: one AD case removed; TrkB-TK-: one AD case removed; TrkB-TK+: one AD removed; Cerebellum: TrkB-Shc: one control and two AD cases removed; TrkB-TK-: one control removed; Protein: Hippocampus: truncated TrkB: one control removed; Temporal cortex: full-length TrkB: one AD case removed; Occipital cortex: full-length TrkB: one AD case removed). Statistical analyses were conducted using STATISTICA 7 (StatSoft Inc., 2000, STATISTICA for Windows). Tests for normality were conducted. For normally distributed data, two-tailed unpaired t-tests were conducted to assess significance in gene expression changes. For data with non-normal distribution, the Mann Whitney U test was

conducted. A one-way analysis of variance (ANOVA) was conducted to assess significance in gene expression changes when incremental treatment concentrations were used. ANOVAs were followed up with the Fischer LSD post-hoc analysis to assess the significance between treatment groups. Pearson's Product Moment correlations were conducted to determine if any relationship existed between TrkB alternative transcript expression and glial fibrillary acidic protein (GFAP) mRNA expression. A p-value less than 0.05 (two-tailed) was considered statistically significant.

Results

TrkB-Shc transcript levels are increased in the hippocampus of AD brains

We found highly significant increases in TrkB-Shc mRNA levels in the hippocampus of AD brains compared to controls ($t=-4.03$, $df=8$, $p=0.004$) (Fig. 2A). A trend towards increased TrkB-TK- mRNA levels was observed in the AD hippocampus compared to controls ($t=-2.10$, $df=8$, $p=0.07$). No significant change in hippocampal TrkB-TK+ mRNA levels could be detected between control and AD cases ($t=-1.26$, $df=9$, $p=0.24$).

To determine whether the increase observed in TrkB-Shc mRNA levels are related to the location and severity of tissue pathology, we assessed whether changes in TrkB-Shc mRNA expression are altered in the temporal cortex (a region affected after the hippocampus) as well as the occipital cortex and cerebellum (two regions less affected in AD). Interestingly, we found no significant change in TrkB-Shc mRNA levels in any of the three brain regions examined between control and AD cases (all $p>0.17$) (Fig. 2B-D). Moreover, no significant differences were observed in TrkB-TK- (all $p>0.16$) or TrkB-TK+ (all $p>0.20$) mRNA levels between control and AD cases.

TrkB protein expression in AD

TrkB immunoreactive bands migrating at ~140kDa (corresponding to full-length TrkB) and ~90kDa (corresponding to truncated TrkB) were detected in all control and AD samples in all four brain regions examined (Fig. 3A). The ~90kDa band appeared large and diffuse in all samples from all four brain regions. This is likely because the TrkB-TK- and TrkB-Shc isoforms are similar in size and both migrate at ~90kDa. We observed significant increases in the ~90kDa TrkB protein in the hippocampus ($t=-2.67$, $df=9$, $p=0.03$) and temporal cortex ($t=-3.60$, $df=15$, $p=0.003$) of AD brains compared to controls (Fig. 3B and C). No significant

difference in expression of the ~90kDa protein was observed between AD and controls in the occipital cortex (U=16, Z=1.68, p=0.09) and cerebellum (t=-1.61, df=10, p=0.14) (Fig. 3D and E).

Interestingly, expression of the ~140kDa band corresponding to the TrkB-TK+ protein was significantly increased in the hippocampus of AD brains compared to controls (U=4, Z=-2.24, p=0.02) (Fig. 3B). No significant differences were observed in expression of the ~140kDa protein in the temporal cortex, occipital cortex, and cerebellum between AD and control brains (Fig. 3C-E) (all p>0.09).

GFAP mRNA levels in AD

Elevations in TrkB-TK- mRNA and truncated TrkB protein levels in AD have previously been associated with increased astrocyte numbers in the diseased state (Duguid et al., 1989, Muramori et al., 1998). To determine if the increases in TrkB-TK- mRNA (trend only) and truncated TrkB protein levels we observed are associated with changes in astrocytes in AD, we measured GFAP mRNA levels in the same brain regions where TrkB alternative transcript levels were assessed. We found elevated levels of GFAP mRNA in the hippocampus (U=5, Z=-2.08, p=0.04), temporal cortex (t=-3.47, df=15, p=0.003), and occipital cortex (U=9, Z=-2.60, p=0.009) of AD brains compared to controls (Fig. S2A-C). No significant change in GFAP transcript levels was observed in the AD cerebellum (t=-0.35, df=8, p=0.73) (Fig. S2D). When we examined whether changes in TrkB-TK- mRNA and truncated protein levels were correlated with changes in GFAP mRNA levels, we found that TrkB-TK- mRNA levels did not correlate with changes in GFAP mRNA levels in any brain region examined. Interestingly, we found significant positive correlations between truncated TrkB protein expression and GFAP mRNA levels in the hippocampus (r=0.89, p=0.0003) and temporal cortex (r=0.51, p=0.04) but no association between truncated TrkB protein and

GFAP mRNA levels in the occipital cortex or cerebellum (all $p>0.27$). GFAP mRNA levels did not correlate with TrkB-Shc, with TrkB-TK+ mRNA levels, or with full-length TrkB protein expression in any brain region examined.

$A\beta_{42}$

We prepared different structural forms of $A\beta_{42}$ using a modified protocol from Ryan et al. (Ryan et al., 2010). The presence of $A\beta_{42}$ monomers, oligomers, and fibrils were confirmed by western blotting (using WO2 antibody which recognizes the Aβ peptide) and found to be consistent with $A\beta_{42}$ preparations previously reported by us and by others (Garzon and Fahnestock, 2007, Kagedal et al., 2010, Ryan et al., 2010) (Fig. 4A). The monomeric $A\beta_{42}$ preparation (lane 1) contained mostly monomers (~4 kDa) but it also contained small oligomers (12-22 kDa). We did not detect any large oligomers or fibrils. The oligomeric $A\beta_{42}$ preparation (lane 2) contained monomers, small and large oligomers (40-70 kDa). The fibrillar $A\beta_{42}$ preparation (lane 3) contained monomers, small oligomers, some large oligomers, and fibrils (>250 kDa).

To extend our characterization of the $A\beta_{42}$ preparations utilized in this study, in addition to western blotting, we also visualized the three different $A\beta_{42}$ preparations using AFM (Fig. 4B). The monomer preparation contained monomers and small aggregates of $A\beta_{42}$. These are likely to correspond to the small oligomers observed in western blot analysis (Fig. 4A lane 1). Also similar to our findings by western blotting, we observed varying sizes of $A\beta_{42}$ oligomers and fibrils in their respective preparations by AFM. In the oligomer preparation, we found evidence of some oligomers aggregating into fibrils (Fig. 4B and S3A). However, it was in the fibril preparation that most fibrillar $A\beta_{42}$ species were observed (Fig.

4B and S3B), including protofibrils which can reach up to ~100 nm in length (Arimon et al., 2005) as indicated by the black arrows in Figure S3B.

Fibrillar Aβ_{42} increases TrkB-Shc transcript levels in the differentiated SHSY5Y neuronal cell-line

The increase in TrkB-Shc mRNA levels in the AD hippocampus but not in other brain regions examined suggests that factors specific to severe AD pathology may be modulating its expression. Thus, we determined whether TrkB alternative transcript expression in neuronal cell-lines can be modulated by different species of Aβ_{42}. We found a modest but significant increase in TrkB-Shc mRNA levels ($t=-2.33$, $df=15$, $p=0.03$) in differentiated SHSY5Y neuronal cells treated with preparations of Aβ_{42} containing fibrils but not with preparations containing monomers or oligomers (all $p>0.36$) (Fig. 5). No significant changes in TrkB-TK- (all $p>0.07$) and TrkB-TK+ (all $p>0.17$) mRNA levels were observed in any of the treatment conditions.

TrkB-Shc co-localizes with TrkB-TK+

TrkB-Shc has previously been shown to co-localize with TrkB-TK+ in non-neuronal cells (Stoilov et al., 2002). To determine whether TrkB-Shc can co-colocalize with TrkB-TK+ in neuronal cells, we co-overexpressed TrkB-Shc and GFP-tagged TrkB-TK+ in differentiated SHSY5Y cells. We observed co-localisation between TrkB-TK+ and TrkB-Shc (Fig. 6). Both TrkB-Shc and TrkB-TK+ showed cytoplasmic and plasma membrane localisation (Fig. 6A, B, and D). Expression of both isoforms could also be observed in neurites. TrkB-TK+ was found to localise mostly around the soma, whereas TrkB-Shc was observed to distribute into more distal regions of neurites. Interestingly, TrkB-TK+ and to a lesser extent, TrkB-Shc,

were found to be highly concentrated in the endoplasmic reticulum. No expression of either isoform could be detected in the nucleus (Fig. 6A-D).

Increased TrkB-Shc expression dampens BDNF-stimulated-TrkB-TK+ mediated second messenger signalling via the MEK pathway

We next determined whether increased TrkB-Shc protein expression impacts on BDNF/TrkB-TK+ signaling in a neuronal setting. Differentiated SHSY5Y cells were transiently transfected with either empty vector or myc-tagged TrkB-Shc plasmids and treated with incremental concentrations of BDNF. In cells transfected with the empty vector plasmid, treatment with exogenous BDNF increased TrkB, ERK1/2, and AKT phosphorylation in a concentration dependent manner (all $F>3.02$, $df=3$, $p<0.02$) (Fig. 7A). In cells transfected with TrkB-Shc-myc, BDNF treatment also increased TrkB, ERK1/2, and AKT phosphorylation (all $F>4.14$, $df=3$, $p<0.03$) (Fig. 7A). No change was observed in total protein levels in the empty vector and TrkB-Shc transfected cells with or without BDNF treatment.

When we examined the ratio of phosphorylated to total (phosphorylated + non-phosphorylated) levels of TrkB, ERK1/2, and AKT as a measure of activity, we found that overexpression of TrkB-Shc in differentiated SHSY5Y cells diminished the BDNF-stimulated increase in pERK1:ERK1 expression ratio (comparing empty vector to TrkB-Shc transfected at 5ng BDNF treatment: $t=2.79$, $df=6$, $p=0.03$; comparing empty vector to TrkB-Shc transfected at 15ng BDNF treatment: $t=2.47$, $df=6$, $p=0.049$) and abolished the BDNF-stimulated increase in pERK2:ERK2 expression ratio compared to cells transfected with the empty vector (empty vector: $F=3.66$, $df=3$, $p=0.04$ compared to TrkB-Shc: $F=0.08$, $df=3$, $p=0.97$) (Fig. 7B). TrkB-Shc overexpression had no significant impact on BDNF-stimulated pAKT:AKT expression ratio compared to empty vector transfected cells. Interestingly, in

both empty vector and TrkB-Shc transfected cells, treatment of cells with 25ng of BDNF had little effect on pAKT:AKT, pERK1:ERK1, and pERK2:ERK2 expression ratios and levels appeared similar to the vehicle treatment condition. This finding suggests that our experimental system was likely to have been saturated at 25ng BDNF. This is likely as treatment with 25ng BDNF had a more potent effect at increasing the pTrkB:TrkB-TK+ expression ratio than in the empty vector ($t=-3.03$, $df=6$, $p=0.02$).

Discussion

In this study, we report several novel findings: First, we found elevated levels of the neuron-specific truncated TrkB alternative transcript, TrkB-Shc in the hippocampus but not in the temporal cortex, occipital cortex, and cerebellum of AD brains. Second, we found that exposure of differentiated SHSY5Y neuronal cell-lines to $A\beta_{42}$ preparations containing fibrils increased TrkB-Shc transcript levels. Finally, increasing TrkB-Shc protein expression in differentiated SHSY5Y cells attenuated BDNF-stimulated-TrkB-TK+ mediated second messenger signaling via the MEK pathway.

TrkB-Shc and AD

To date, TrkB-Shc expression in AD brain tissues has yet to be described. Considering that TrkB-Shc expression is neuron-specific (Stoilov et al., 2002), it is surprising that this gap in the knowledgebase exists. Here, we show that TrkB-Shc transcripts are elevated in the AD hippocampus, but not in the temporal cortex, occipital cortex, or cerebellum; suggesting that increases in this TrkB alternative splice transcript is occurring in brain regions that are most severely affected in the diseased state and that the observed increase may likely be influenced by the neuronal cell population. In support of this, we found that TrkB-Shc mRNA levels in differentiated neuronal cell-lines can be increased by exposure to preparations of $A\beta_{42}$ containing fibrils. Interestingly, $A\beta_{42}$ preparations that did not contain fibrillar $A\beta_{42}$ species had no significant effect on TrkB-Shc transcript levels.

At present, it is widely accepted that soluble oligomers are the more neurotoxic of the $A\beta_{42}$ species, however, evidence also indicates that the neurotoxicity of $A\beta_{42}$ requires its aggregation in the fibrillar form, particularly in the form of protofibrils (Walsh et al., 1999, Serpell, 2000). By AFM, we were able to detect various sizes of fibrils in the fibril $A\beta_{42}$ preparations, including protofibrils which can be ~100 nm in length. Our images of the fibril

preparations are very similar to those reported by Arimon et al. (2005). Here, the authors found that protofibrils co-exist with larger fibrils and are able to incorporate into larger fibrillar structures. In our preparations, we observed similar finidings. In the oligomer preparations, while we observed evidence of some oligomers aggregating into fibrils, the predominant form of $A\beta_{42}$ were globular oligomers of various sizes. Considering that $A\beta_{42}$ fibrils are formed in the advanced stages of AD (Hoozemans et al., 2006), that the hippocampus is the most impacted brain region, and that the CA1 subregion (the hippocampal region assessed in this study) is the most severely impacted subregion of the hippocampus in AD, altogether, our findings suggest that the selective increase in the TrkB-Shc alternative splice transcript in the AD hippocampus may be specific to severe, late stage pathology.

In the AD hippocampus, we found a trend towards an increase in TrkB-TK- mRNA levels but a significant elevation in truncated TrkB protein. Both TrkB-TK- and TrkB-Shc are predicted to migrate at similar molecular weights by denaturing SDS-PAGE, thus the increase in truncated TrkB protein is likely to reflect an increase in both TrkB-TK- and TrkB-Shc protein in the total hippocampal brain homogenate. While an increase in TrkB-TK- mRNA levels and truncated TrkB protein are consistent with previous findings (Connor et al., 1996, Ferrer et al., 1999), it is in contrast to the lack of change we observed in differentiated SHSY5Y cells exposed to $A\beta_{42}$. TrkB-TK- is primarily expressed by astrocytes and previous studies have demonstrated that TrkB-TK- signaling via Rho-GTPase is critical in regulating astrocyte morphology (Ohira et al., 2005a). Considering that astrocytes become activated when exposed to Aβ, the increase in TrkB-TK- in the AD hippocampus may be more reflective of increases in astrocytic TrkB-TK- expression as opposed to neuronal TrkB-TK- expression. This is likely as elevations in TrkB-TK- mRNA in AD have previously been found to be associated with reactive glia around senile plaques (Ferrer et al., 1999).

Moreover, when we surveyed GFAP mRNA levels in the hippocampus, temporal cortex, occipital cortex, and cerebellum, we found statistically significant elevations in all brain regions except for the cerebellum (a region relatively spared in AD).

Interestingly, while we found no significant changes in TrkB-TK+ mRNA levels in any of the four brain regions tested in AD compared to controls, we did find a significant increase in full-length TrkB protein in the hippocampus. This is consistent with previous findings which showed that both truncated and full-length TrkB immunoreactivity in the CA1 subregion of the hippocampus are elevated in AD compared to controls (Connor et al., 1996). Moreover, both truncated and full-length TrkB immunoreactivity detected by Connor et al. (1996) was found to localise to glial cells in association with senile plaques. Considering that astrocytes are also able to express TrkB-TK+ mRNA and full-length TrkB protein, our finding of elevated full-length TrkB protein in the AD hippocampus suggests that the increase is likely to be contributed by astrocytes.

In addition to the hippocampus, we also found a significant increase in truncated TrkB protein in the temporal cortex but not in the occipital cortex and cerebellum, consistent with previous findings (Allen et al., 1999). The occipital cortex is affected less severely in AD and the cerebellum is relatively spared. If we take into consideration our finding of increased full-length TrkB protein in the AD hippocampus, and findings of elevated GFAP mRNA levels in the hippocampus and temporal and occipital cortices, altogether, our findings may also be reflective of the progression of AD brain pathology ie. where pathology is most severe in the hippocampus>temporal cortex>occipital cortex>cerebellum.

Increased TrkB-Shc in AD: a negative regulator of BDNF/TrkB-TK+ signaling in AD?

The cellular response to BDNF is mediated by the full-length TrkB receptor, TrkB-TK+, and this can be modulated depending upon the relative ratios of the full-length TrkB to truncated

TrkB receptors (Eide et al., 1996). Changes in the relative levels of truncated TrkB receptors have been demonstrated to have a significant impact on neuronal BDNF availability and signaling. Co-expression of TrkB-TK- and TrkB-Shc in vitro have been found to have a dominant negative effect on TrkB-TK+ activity (Biffo et al., 1995, Eide et al., 1996, Ninkina et al., 1996, Stoilov et al., 2002). In accordance with these findings, we showed that TrkB-Shc can have a negative impact on human neuronal BDNF/TrkB-TK+ second messenger signaling via the MEK pathway where BDNF/TrkB-TK+ mediated phosphoryation of both ERK1 and ERK2 were reduced when exogenous TrkB-Shc was overexpressed. In contrast, little change was obseved in BDNF-stimulated phosphorylation of TrkB and AKT when TrkB-Shc was overexpressed. Unlike TrkB-TK+ signaling via the MEK pathway, which requires Shc adaptor proteins to bind to phosphorylated tyrosine residues on TrkB-TK+, signaling via AKT can also mediated via IRS1/2 (Yamada et al., 1997). Thus, the lack of change in AKT phosphorylation upon TrkB-Shc overexpression may have resulted from alternative pathways being utilized upon BDNF stimulation.

Increased TrkB-Shc in AD: a compensatory response to promote cell survival in AD?

While there is evidence to support the notion that increased TrkB-Shc will negatively impact neuronal BDNF signaling (Biffo et al., 1995, Eide et al., 1996, Ninkina et al., 1996, Stoilov et al., 2002), recent evidence suggests that TrkB-Shc may play a protective role in AD by promoting cell survival. It was recently demonstrated that overexpression of TrkB-Shc can modulate APP metabolism by decreasing APP intracellular domain (ICD)-mediated transcription (AICD) (Ansaloni et al., 2011). The AICD is a positive transcriptional regulator of apoptosis and is suggested to further accelerate neurodegeneration by contributing to the toxic effect of $A\beta_{42}$ aggregates in vivo.

In our current study, we found that overexpression of TrkB-Shc in differentiated SHSY5Y cells could blunt BDNF/TrkB-TK+ stimulated MEK pathway signaling by attenuating pERK1/2:ERK1/2 ratios (a measure of ERK1/2 activity) but with little effect on pTrkB:TrkB-TK+ and pAKT:AKT ratios. In AD, MEK signaling and ERK1/2 phosphorylation is increased in vulnerable neurons, moreover, elevated MEK pathway activity is implicated in the abnormal phosphorylation of tau and neurofilament proteins (Perry et al., 1999). Considering that BDNF/TrkB-TK+ mediated neuronal survival is more dependent upon AKT signaling than MEK signaling (Atwal et al., 2000), our finding that elevated levels of TrkB-Shc in a neuronal setting can attenuate ERK1/2 phosphorylation following BDNF stimulation (but have no significant effect on phosphorylated AKT and TrkB at non-saturating levels) suggests that the increase in TrkB-Shc transcripts in the AD hippocampus and when cells are treated with fibrillar $A\beta_{42}$, may function as a compensatory mechanism in neurons in AD to promote survival. Further work is required to determine how TrkB-Shc is functioning in neurons in AD and whether changes in their expression at different stages of the disease process, in response to Aβ, or in response to other amyloidogenic factors may be protective or deleterious.

Conclusions

In this study, we demonstrated a novel increase in TrkB-Shc transcript levels in the AD hippocampus and present evidence to suggest that the increased expression of this TrkB alternative splice transcript may be induced by exposure of neuronal cells to fibrillar $A\beta_{42}$ species. Our finding that TrkB-Shc can preferentially dampen BDNF-stimulated TrkB-TK+ second messenger signaling via the MEK pathway but not the AKT pathway provides compelling evidence to suggest that increases in TrkB-Shc may have a protective role in promoting cell survival pathways but without further activating an already overactive MEK

signaling pathway in AD. Further work aimed at identifying how splicing of the TrkB alternative transcripts in neurons is modulated in AD would be an important step in understanding how TrkB-Shc expression is modulated in AD.

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Conflict of Interest

There are no conflicts of interest

References

Allen SJ, Wilcock GK, Dawbarn D (1999), Profound and selective loss of catalytic TrkB immunoreactivity in Alzheimer's disease. Biochem Biophys Res Commun 264:648-651.

Alonso M, Vianna MR, Depino AM, Mello e Souza T, Pereira P, Szapiro G, Viola H, Pitossi F, Izquierdo I, Medina JH (2002), BDNF-triggered events in the rat hippocampus are required for both short- and long-term memory formation. Hippocampus 12:551-560.

Ansaloni S, Leung BP, Sebastian NP, Samudralwar R, Gadaleta M, Saunders AJ (2011), TrkB Isoforms Differentially Affect AICD Production through Their Intracellular Functional Domains. Int J Alzheimers Dis 2011:729382.

Arimon M, Diez-Perez I, Kogan MJ, Durany N, Giralt E, Sanz F, Fernandez-Busquets X (2005), Fine structure study of Abeta1-42 fibrillogenesis with atomic force microscopy. Faseb J 19:1344-1346.

Atwal JK, Massie B, Miller FD, Kaplan DR (2000), The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. Neuron 27:265-277.

Bartkowska K, Turlejski K, Djavadian RL (2010), Neurotrophins and their receptors in early development of the mammalian nervous system. Acta Neurobiol Exp (Wars) 70:454-467.

Beck KD, Lamballe F, Klein R, Barbacid M, Schauwecker PE, McNeill TH, Finch CE, Hefti F, Day JR (1993), Induction of noncatalytic TrkB neurotrophin receptors during axonal sprouting in the adult hippocampus. J Neurosci 13:4001-4014.

Biffo S, Offenhauser N, Carter BD, Barde YA (1995), Selective binding and internalisation by truncated receptors restrict the availability of BDNF during development. Development 121:2461-2470.

Connor B, Young D, Lawlor P, Gai W, Waldvogel H, Faull RL, Dragunow M (1996), Trk receptor alterations in Alzheimer's disease. Brain Res Mol Brain Res 42:1-17.

Duguid JR, Bohmont CW, Liu NG, Tourtellotte WW (1989), Changes in brain gene expression shared by scrapie and Alzheimer disease. Proc Natl Acad Sci U S A 86:7260-7264.

Eide FF, Vining ER, Eide BL, Zang K, Wang XY, Reichardt LF (1996), Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J Neurosci 16:3123-3129.

Ferrer I, Marin C, Rey MJ, Ribalta T, Goutan E, Blanco R, Tolosa E, Marti E (1999), BDNF and full-length and truncated TrkB expression in Alzheimer disease. Implications in therapeutic strategies. J Neuropathol Exp Neurol 58:729-739.

Frisen J, Verge VM, Fried K, Risling M, Persson H, Trotter J, Hokfelt T, Lindholm D (1993), Characterization of glial trkB receptors: differential response to injury in the central and peripheral nervous systems. Proc Natl Acad Sci U S A 90:4971-4975.

Fryer RH, Kaplan DR, Kromer LF (1997), Truncated trkB receptors on nonneuronal cells inhibit BDNF-induced neurite outgrowth in vitro. Exp Neurol 148:616-627.

Gao X, Chen J (2009), Conditional knockout of brain-derived neurotrophic factor in the hippocampus increases death of adult-born immature neurons following traumatic brain injury. J Neurotrauma 26:1325-1335.

Gao X, Smith GM, Chen J (2009), Impaired dendritic development and synaptic formation of postnatal-born dentate gyrus granular neurons in the absence of brain-derived neurotrophic factor signaling. Exp Neurol 215:178-190.

Garzon DJ, Fahnestock M (2007), Oligomeric amyloid decreases basal levels of brain-derived neurotrophic factor (BDNF) mRNA via specific downregulation of BDNF

transcripts IV and V in differentiated human neuroblastoma cells. J Neurosci 27:2628-2635.

Ghosh A, Carnahan J, Greenberg ME (1994), Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263:1618-1623.

Gregory GC, Macdonald V, Schofield PR, Kril JJ, Halliday GM (2006), Differences in regional brain atrophy in genetic forms of Alzheimer's disease. Neurobiol Aging 27:387-393.

Higgins MJ, Sader JE, Jarvis SP (2006), Frequency modulation atomic force microscopy reveals individual intermediates associated with each unfolded I27 titin domain. Biophys J 90:640-647.

Hoozemans JJ, Chafekar SM, Baas F, Eikelenboom P, Scheper W (2006), Always around, never the same: pathways of amyloid beta induced neurodegeneration throughout the pathogenic cascade of Alzheimer's disease. Curr Med Chem 13:2599-2605.

Itami C, Mizuno K, Kohno T, Nakamura S (2000), Brain-derived neurotrophic factor requirement for activity-dependent maturation of glutamatergic synapse in developing mouse somatosensory cortex. Brain Res 857:141-150.

Kagedal K, Kim WS, Appelqvist H, Chan S, Cheng D, Agholme L, Barnham K, McCann H, Halliday G, Garner B (2010), Increased expression of the lysosomal cholesterol transporter NPC1 in Alzheimer's disease. Biochim Biophys Acta 1801:831-838.

Kim WS, Bhatia S, Elliott DA, Agholme L, Kagedal K, McCann H, Halliday GM, Barnham KJ, Garner B (2010), Increased ATP-binding cassette transporter A1 expression in Alzheimer's disease hippocampal neurons. J Alzheimers Dis 21:193-205.

Klein R, Conway D, Parada LF, Barbacid M (1990), The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 61:647-656.

Klein R, Nanduri V, Jing SA, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991), The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66:395-403.

Knusel B, Winslow JW, Rosenthal A, Burton LE, Seid DP, Nikolics K, Hefti F (1991), Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. Proc Natl Acad Sci U S A 88:961-965.

Livak KJ, Schmittgen TD (2001), Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Lu Y, Christian K, Lu B (2008), BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? Neurobiol Learn Mem 89:312-323.

Luberg K, Wong J, Weickert CS, Timmusk T (2010), Human TrkB gene: novel alternative transcripts, protein isoforms and expression pattern in the prefrontal cerebral cortex during postnatal development. J Neurochem 113:952-964.

McAllister AK, Lo DC, Katz LC (1995), Neurotrophins regulate dendritic growth in developing visual cortex. Neuron 15:791-803.

Mizuno M, Yamada K, Olariu A, Nawa H, Nabeshima T (2000), Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. J Neurosci 20:7116-7121.

Muramori F, Kobayashi K, Nakamura I (1998), A quantitative study of neurofibrillary tangles, senile plaques and astrocytes in the hippocampal subdivisions and entorhinal cortex in Alzheimer's disease, normal controls and non-Alzheimer neuropsychiatric diseases. Psychiatry Clin Neurosci 52:593-599.

NIA (1997), Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. The National Institute on Aging, and Reagan Institute Working Group on

Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease. Neurobiol Aging 18:S1-2.

Ninkina N, Adu J, Fischer A, Pinon LG, Buchman VL, Davies AM (1996), Expression and function of TrkB variants in developing sensory neurons. Embo J 15:6385-6393.

Ohira K, Hayashi M (2003), Expression of TrkB subtypes in the adult monkey cerebellar cortex. J Chem Neuroanat 25:175-183.

Ohira K, Kumanogoh H, Sahara Y, Homma KJ, Hirai H, Nakamura S, Hayashi M (2005a), A truncated tropomyosin-related kinase B receptor, T1, regulates glial cell morphology via Rho GDP dissociation inhibitor 1. J Neurosci 25:1343-1353.

Ohira K, Shimizu K, Yamashita A, Hayashi M (2005b), Differential expression of the truncated TrkB receptor, T1, in the primary motor and prefrontal cortices of the adult macaque monkey. Neurosci Lett 385:105-109.

Patapoutian A, Reichardt LF (2001), Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 11:272-280.

Perry G, Roder H, Nunomura A, Takeda A, Friedlich AL, Zhu X, Raina AK, Holbrook N, Siedlak SL, Harris PL, Smith MA (1999), Activation of neuronal extracellular receptor kinase (ERK) in Alzheimer disease links oxidative stress to abnormal phosphorylation. Neuroreport 10:2411-2415.

Ryan DA, Narrow WC, Federoff HJ, Bowers WJ (2010), An improved method for generating consistent soluble amyloid-beta oligomer preparations for in vitro neurotoxicity studies. J Neurosci Methods 190:171-179.

Savaskan E, Muller-Spahn F, Olivieri G, Bruttel S, Otten U, Rosenberg C, Hulette C, Hock C (2000), Alterations in trk A, trk B and trk C receptor immunoreactivities in parietal cortex and cerebellum in Alzheimer's disease. Eur Neurol 44:172-180.

Schindowski K, Belarbi K, Buee L (2008), Neurotrophic factors in Alzheimer's disease: role of axonal transport. Genes Brain Behav 7 Suppl 1:43-56.

Serpell LC (2000), Alzheimer's amyloid fibrils: structure and assembly. Biochim Biophys Acta 1502:16-30.

Stoilov P, Castren E, Stamm S (2002), Analysis of the human TrkB gene genomic organization reveals novel TrkB isoforms, unusual gene length, and splicing mechanism. Biochem Biophys Res Commun 290:1054-1065.

Takahashi RH, Almeida CG, Kearney PF, Yu F, Lin MT, Milner TA, Gouras GK (2004), Oligomerization of Alzheimer's beta-amyloid within processes and synapses of cultured neurons and brain. J Neurosci 24:3592-3599.

Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condron MM, Lomakin A, Benedek GB, Selkoe DJ, Teplow DB (1999), Amyloid beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. J Biol Chem 274:25945-25952.

Wong J, Rothmond DA, Webster MJ, Weickert CS (2011), Increases in Two Truncated TrkB Isoforms in the Prefrontal Cortex of People with Schizophrenia Schizophr Bull Epub.

Wong J, Webster MJ, Cassano H, Weickert CS (2009), Changes in alternative brain-derived neurotrophic factor transcript expression in the developing human prefrontal cortex. Eur J Neurosci 29:1311-1322.

Yamada M, Ohnishi H, Sano S, Nakatani A, Ikeuchi T, Hatanaka H (1997), Insulin receptor substrate (IRS)-1 and IRS-2 are tyrosine-phosphorylated and associated with phosphatidylinositol 3-kinase in response to brain-derived neurotrophic factor in cultured cerebral cortical neurons. J Biol Chem 272:30334-30339.

Figure Legends

Table 1. Cohort Demographics

Table 2. Primer Sequences

Fig. 1. TrkB gene and protein structure

In the upper panel, exons are represented by numbered rectangles, lines joining exons depict exon splicing. Colored exons correspond to protein domains in lower panel. White exons represent non-coding sequences. Nomenclature and gene organization derived from Luberg et al. (Luberg et al., 2010) and Wong et al. (Wong et al., 2011).

Fig. 2. TrkB alternative transcript expression in AD

Expression of TrkB alternative transcripts in the (A) hippocampus, (B) temporal cortex, (C) occipital cortex, and (D) cerebellum of control (white) and AD (black) brains were measured by qPCR. **=p=0.004; #=p=0.07.

Fig. 3. TrkB protein expression in AD

Expression of truncated (90 kDa) and full-length (140 kDa) TrkB protein in the (A) hippocampus, (B) temporal cortex, (C) occipital cortex, and (D) cerebellum of control (white) and AD (black) brains were measured by western blotting. **=p=0.003; *=p=0.02 (140 kDa band) and p=0.03 (90 kDa band).

Fig. 4. Aβ_{42} preparations

(A) Lane 1 is predominantly monomers and also includes some small oligomers. Lane 2 consists of large oligomers (40-70 kDa) which are absent in lanes 1. Lane 3 consists of fibrils (>250 kDa) which are absent in lanes 1 and 2. (B) Atomic force microscopy images of the monomer (lane 1), oligomer (lane 2), and fibril (lane 3) preparations from panel (A). (i) The monomer preparations contained monomers. Aggregation of monomers also can be seen. (ii) The oligomer preparation contained monomers and oligomers of various sizes. Aggregation of oligomers into fibrils is evident. (iii) The fibril preparation contained $A\beta_{42}$ fibrils of various sizes. Scale represents 1 μm.

Fig. 5. Effect of different structural forms of $A\beta_{42}$ on TrkB alternative transcript expression in the differentiated SHSY5Y neuronal cell-line

SHSY5Y cells were differentiated for 9 days and incubated in the absence (white bars) or presence of $A\beta_{42}$ monomers (AβM) (grey bars), oligomers (AβO) (black bars), and fibrils (AβF) (hatched bars) for 6 h and harvested. Expression of TrkB alternative transcripts were then measured by qPCR. Data is expressed as mean + SEM relative to the control condition which was set to 1. *=p=0.03. Representative of n=6 separate experiments.

Fig. 6. Localization of TrkB-TK+ and TrkB-Shc in differentiated SHSY5Y neuronal cell-line

Differentiated SHSY5Y (A-D) cells were transfected with TrkB-TK+-EGFP or TrkB-Shc-myc for 24 h. Cells were fixed and immunoprobed with myc antibody for TrkB-Shc protein expression (green for TrkB-TK+EGFP and red for myc tagged TrkB-Shc) and stained with DAPI (blue). Note: no colocalization with DAPI (blue) was observed in either the green (TrkB-TK+-EGFP) or red (myc-tagged TrkB-Shc) channels. Representative of n=3 separate experiments.

Fig. 7. Effect of TrkB-Shc overexpression on BDNF-stimulated TrkB-K+ second messenger signaling in differentiated SHSY5Y neuronal cell-line

Differentiated SHSY5Y cells were transfected with either empty vector (EV; blue) or TrkB-Shc-myc (pink) for 24 h and treated with increasing concentrations of BDNF for 15 minutes and harvested. Proteins were separated by SDS-PAGE and immunoprobed. (A) Representative western blot images from n=4 independent experiments. (B) Bands were quantitated by densitometry and presented as protein expression ratios of phosphorylated protein to non-phosphorylated protein (pTrkB-TK+:TRkB-TK+; pAKT:AKT; pERK1:ERK1; pERK2:ERK2) -/+ SEM. *=p=0.02 for pTrkB:TrkB-TK+ at 25ng, p=0.03 for pERK1:ERK1 at 5ng BDNF and p=0.049 for pERK1:ERK1 at 15ng BDNF. Representative of n=4 separate experiments.

Fig. S1. β-Actin mRNA expression in AD

(A) CT values of β-actin from qPCR of the hippocampus (HP), temporal cortex (TC), occipital cortex (OC), and cerebellum (CB) of control (white) and AD (black) brains. (B) β-Actin mRNA levels was normalized to the geometric mean calculated from β-actin, cyclophillin, and porphobilinogen deaminase to show that β-actin mRNA levels is not affected by diagnosis.

Fig. S2. GFAP mRNA expression in AD

Expression of GFAP mRNA in the (A) hippocampus (HP), (B) temporal cortex (TC), (C) occipital cortex (OC), and (D) cerebellum (CB) of control (white) and AD (black) brains was measured by qPCR. **=p=0.003 (temporal cortex) and p=0.009 (occipital cortex); *=p=0.04 (hippocampus).

Fig. S3. Atomic force microscopy of $A\beta_{42}$ species

Magnified AFM images of the (A) oligomer and (B) fibril preparations from Fig. 4. Scale represents 0.5 μm for (A) and 0.2 μm for (B). Black arrows in (B) indicate protofibrils which can reach up to ~100 nm in length.

Table 1. Cohort Demographics

Diagnosis	Regions	Gender	Age (years)	PMI (h)
Control	hippocampus, cerebellum	Male	82	23.5
Control	hippocampus, cerebellum	Female	78	11
Control	hippocampus, cerebellum	Male	69	13.5
Control	hippocampus, cerebellum	Female	93	21
Control	hippocampus, cerebellum	Female	85	23
Control	hippocampus, cerebellum	Male	79	8
Control	occipital, temporal	Female	61	26
Control	occipital, temporal	Male	69	48
Control	occipital, temporal	Female	73	44
Control	occipital, temporal	Male	75	60
Control	occipital, temporal	Female	77	48
Control	occipital, temporal	Female	83	28
Control	occipital, temporal	Female	91	40
Control	occipital, temporal	Female	93	17
AD	hippocampus, cerebellum	Male	83	27
AD	hippocampus, cerebellum	Female	84	9
AD	hippocampus, cerebellum	Female	83	23
AD	hippocampus, cerebellum	Male	73	20
AD	hippocampus, cerebellum	Female	83	7
AD	hippocampus, cerebellum	Male	69	15
AD	occipital, temporal	Female	65	36
AD	occipital, temporal	Male	67	38
AD	occipital, temporal	Female	68	36
AD	occipital, temporal	Male	71	36
AD	occipital, temporal	Female	75	76
AD	occipital, temporal	Female	78	24
AD	occipital, temporal	Female	78	43
AD	occipital, temporal	Female	80	48
AD	occipital, temporal	Male	83	30
AD	occipital, temporal	Male	90	9

AD=Alzheimer's disease; PMI=postmortem interval

Table 2. Primer Sequences

Target Gene	GenBank Accession	Forward Sequence	Reverse Sequence	Primer Tm	PCR Product Size (bp)	Reference
TrkB-Shc	AF410901	AGCTCAAGCCAGACACATG	TTAGTCATTAGAGCACACTGC	56	246	Present Study
TrkB-TK-	NM_001007097	TTGGCAAGACACTCCAAGTT	TCACAGCTCACAGTATATGC	54	256	Present Study
TrkB-TK+	NM_001018064	TGGTCTTTGAGTACATGAAGC	TCCTGTACATGATGCTCTCTG	58	342	Present Study
GFAP	NM_002055	AATGCTGGCTTCAAGGAGACC	TTGTCCCTCTCAACCTCCAG	64	254	Present Study
β-Actin	NM_001101	GAATTCTGGCCACGGCTGCTTCCAGCT	AAGCTTTTTCGTGGATGCCACAGGACT	60	162	Kim et al. 2010

Fig. 1.

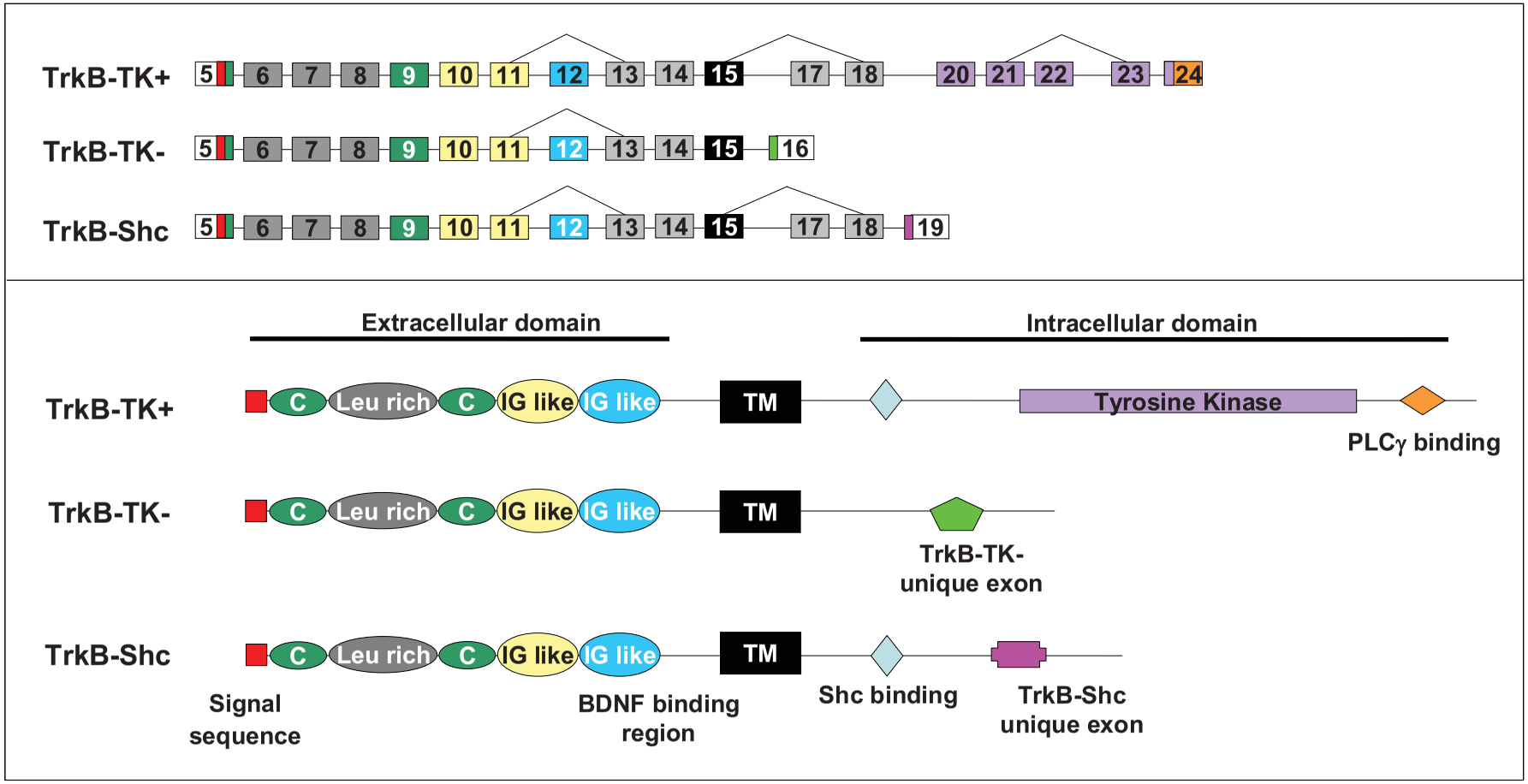

TrkB-TK+
5 6 7 8 9 10 11 12 13 14 15 17 18 20 21 22 23 24
TrkB-TK-
5 6 7 8 9 10 11 12 13 14 15 16
TrkB-Shc
5 6 7 8 9 10 11 12 13 14 15 17 18 19
Extracellular domain
Intracellular domain
TrkB-TK+
C Leu rich C IG like IG like
TM
Tyrosine Kinase
PLCγ binding
TrkB-TK-
C Leu rich C IG like IG like
TM
TrkB-TK-
unique exon
TrkB-Shc
C Leu rich C IG like IG like
TM
Signal
sequence
BDNF binding
region
Shc binding
TrkB-Shc
unique exon

Fig. 2.

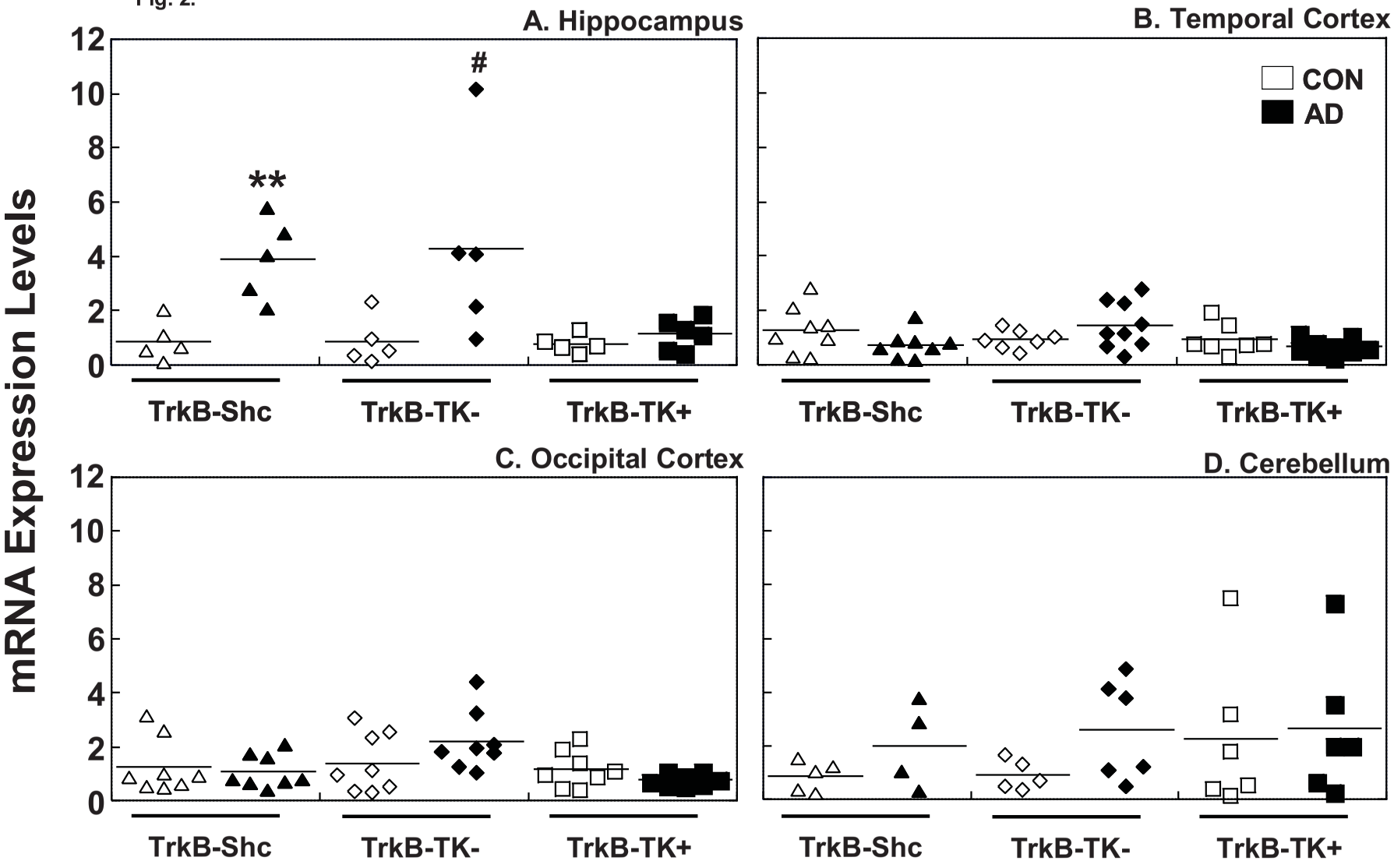

A. Hippocampus
B. Temporal Cortex
C. Occipital Cortex
D. Cerebellum
mRNA Expression Levels
CON
AD
**
#
0
2
4
6
8
10
12
TrkB-Shc
TrkB-TK-
TrkB-TK+

Fig. 3.

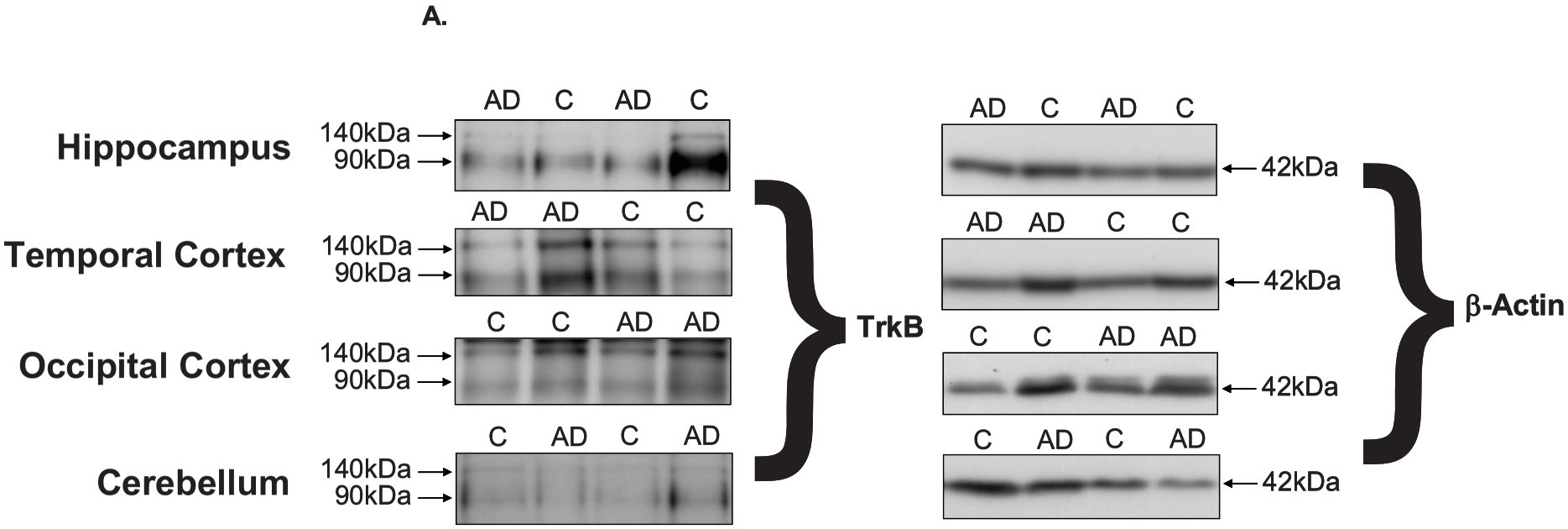

A.
Hippocampus
Temporal Cortex
Occipital Cortex
Cerebellum
AD C AD C
AD AD C C
C C AD AD
C AD C AD
140kDa
90kDa
TrkB
42kDa
β-Actin

Fig. 3. Cont'd

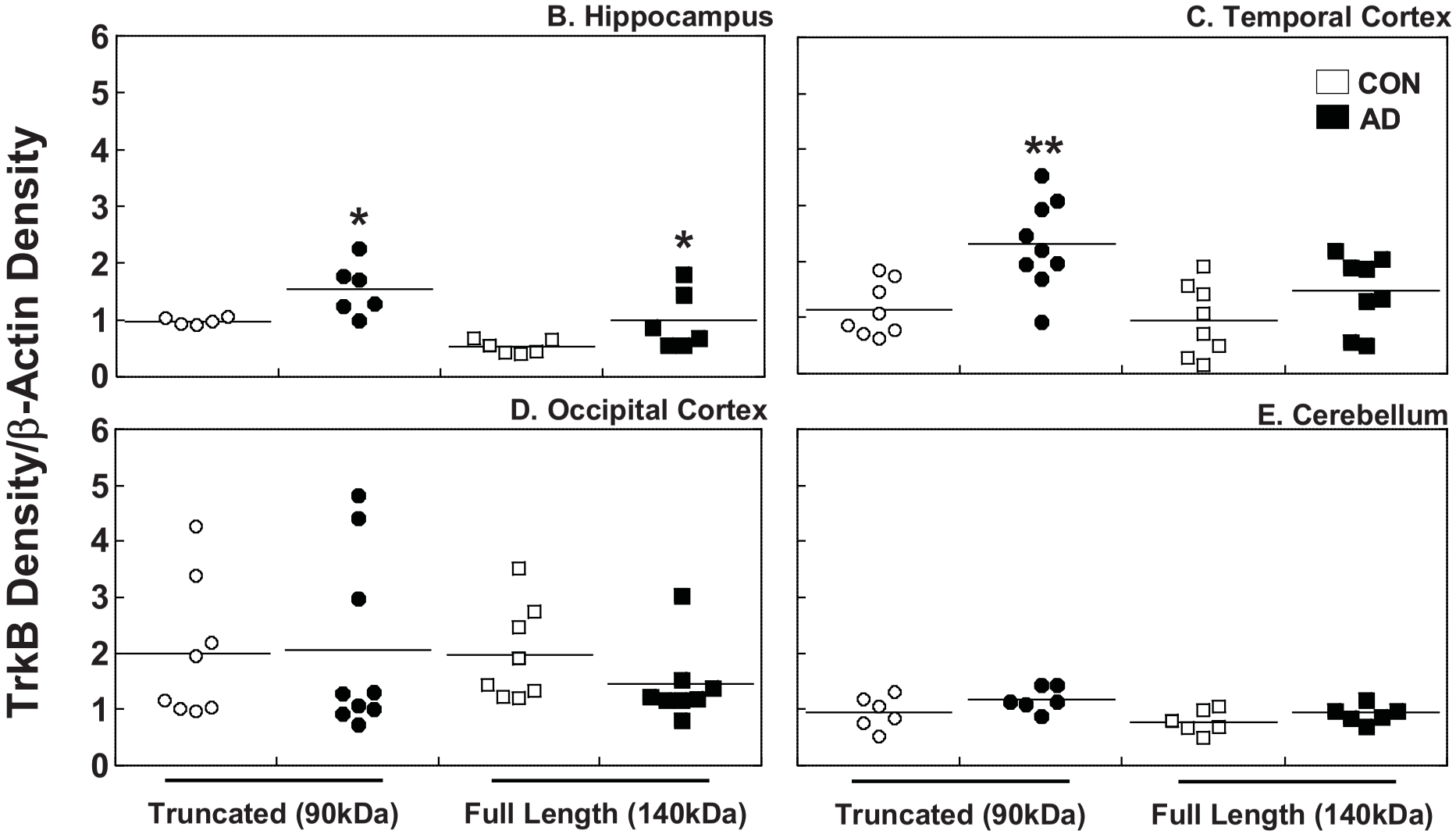
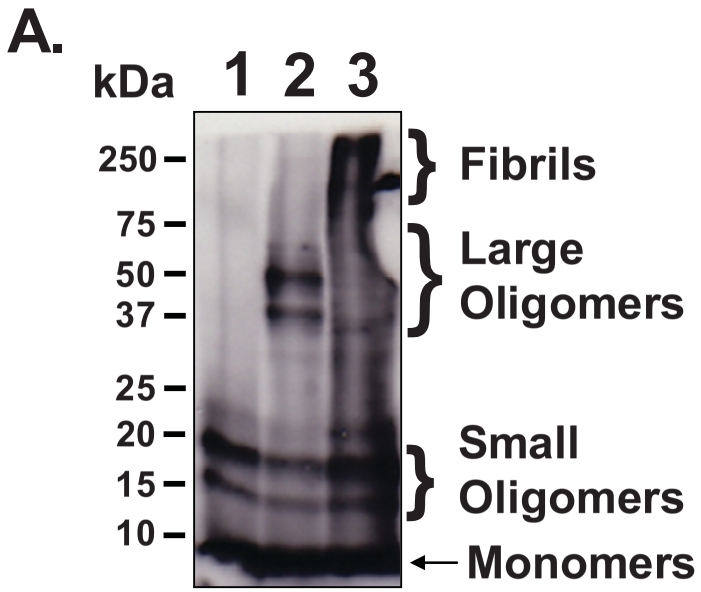

B. Hippocampus
C. Temporal Cortex
D. Occipital Cortex
E. Cerebellum
CON
AD
TrkB Density/β-Actin Density
0
1
2
3
4
5
6
*
*
**
Truncated (90kDa)
Full Length (140kDa)
Truncated (90kDa)
Full Length (140kDa)

Fig. 4.


A.
kDa
1 2 3
250
75
50
37
25
20
15
10
Fibrils
Large Oligomers
Small Oligomers
Monomers

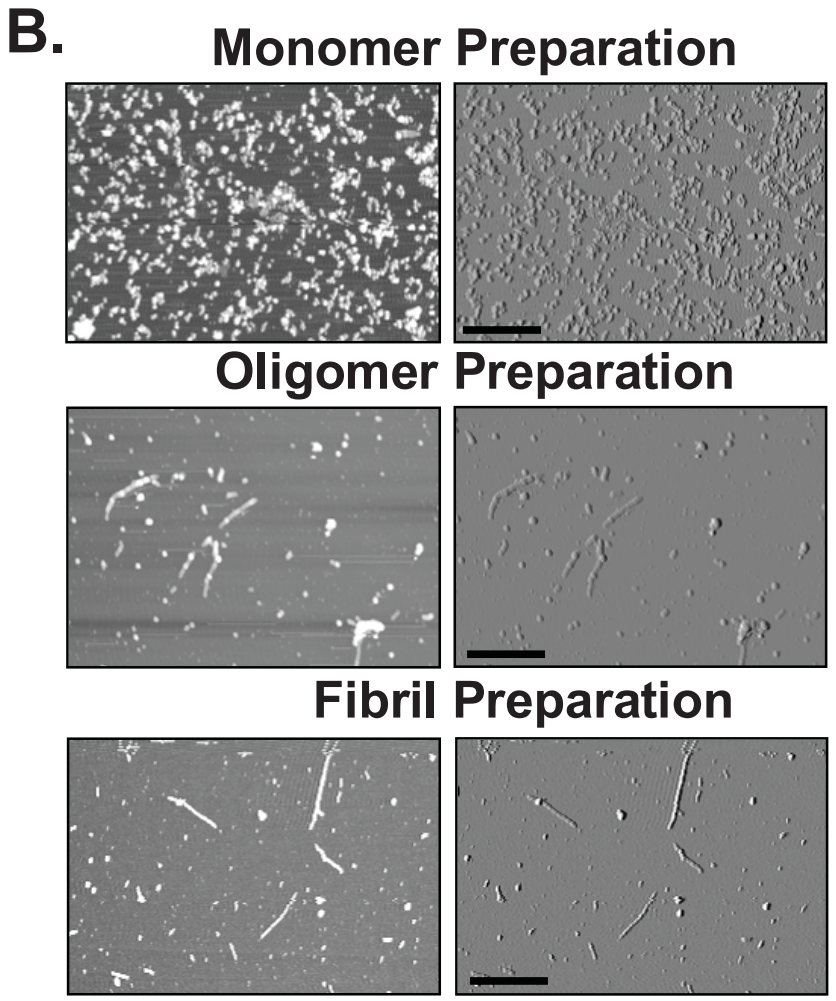

B.
Monomer Preparation
Oligomer Preparation
Fibril Preparation

Fig. 5.

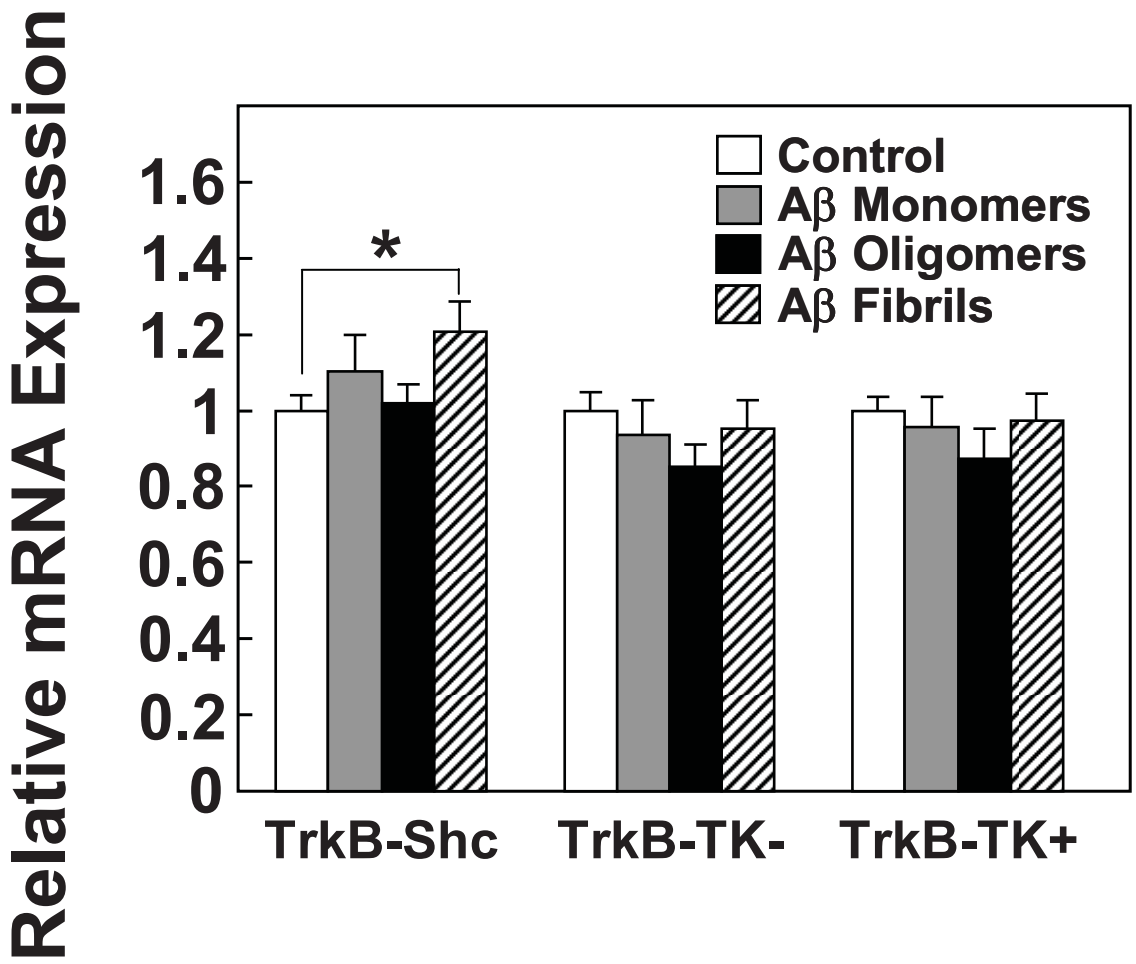

Relative mRNA Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Aβ Monomers
Aβ Oligomers
Aβ Fibrils
TrkB-Shc
TrkB-TK-
TrkB-TK+

Fig. 6.

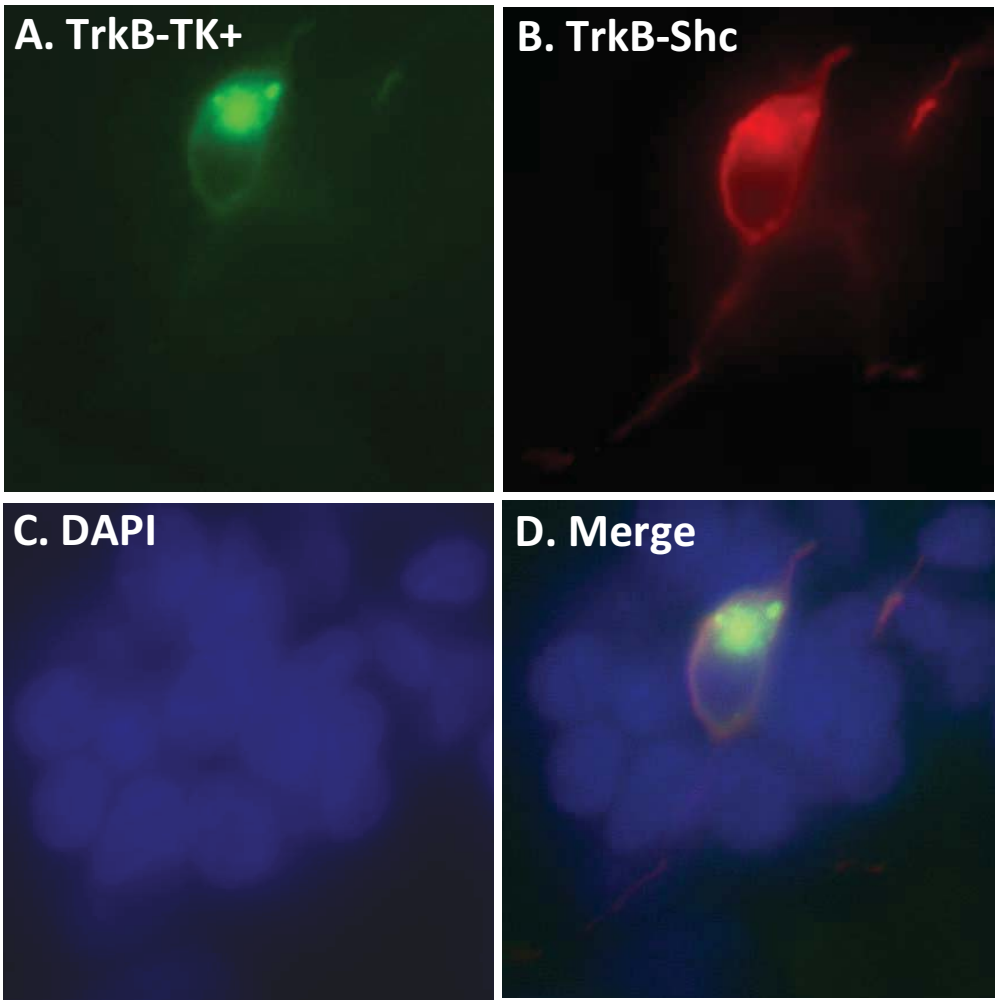


Fig. 7.

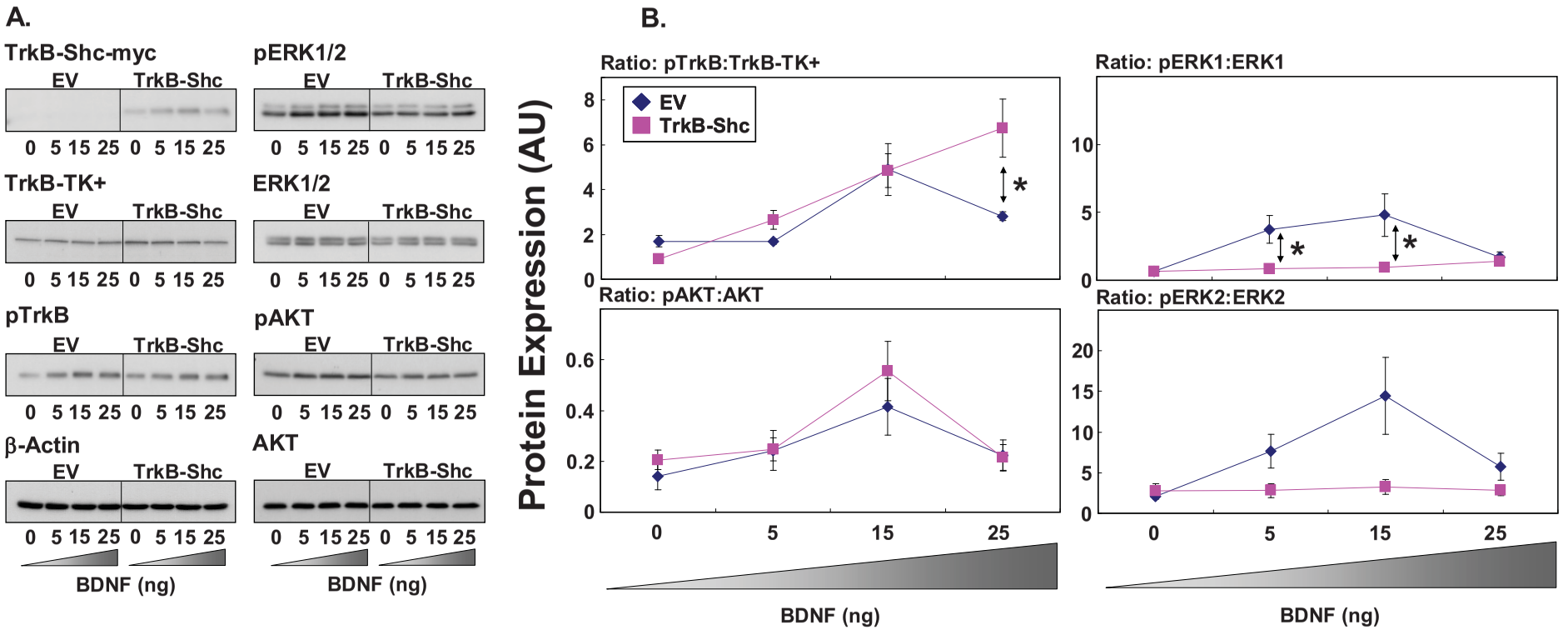

A.
TrkB-Shc-myc
pERK1/2
TrkB-TK+
ERK1/2
pTrkB
pAKT
β-Actin
AKT
EV
TrkB-Shc
0 5 15 25 0 5 15 25
BDNF (ng)
B.
Protein Expression (AU)
Ratio: pTrkB:TrkB-TK+
Ratio: pERK1:ERK1
Ratio: pAKT:AKT
Ratio: pERK2:ERK2
EV
TrkB-Shc
*
0 5 15 25
BDNF (ng)

Fig. S1.

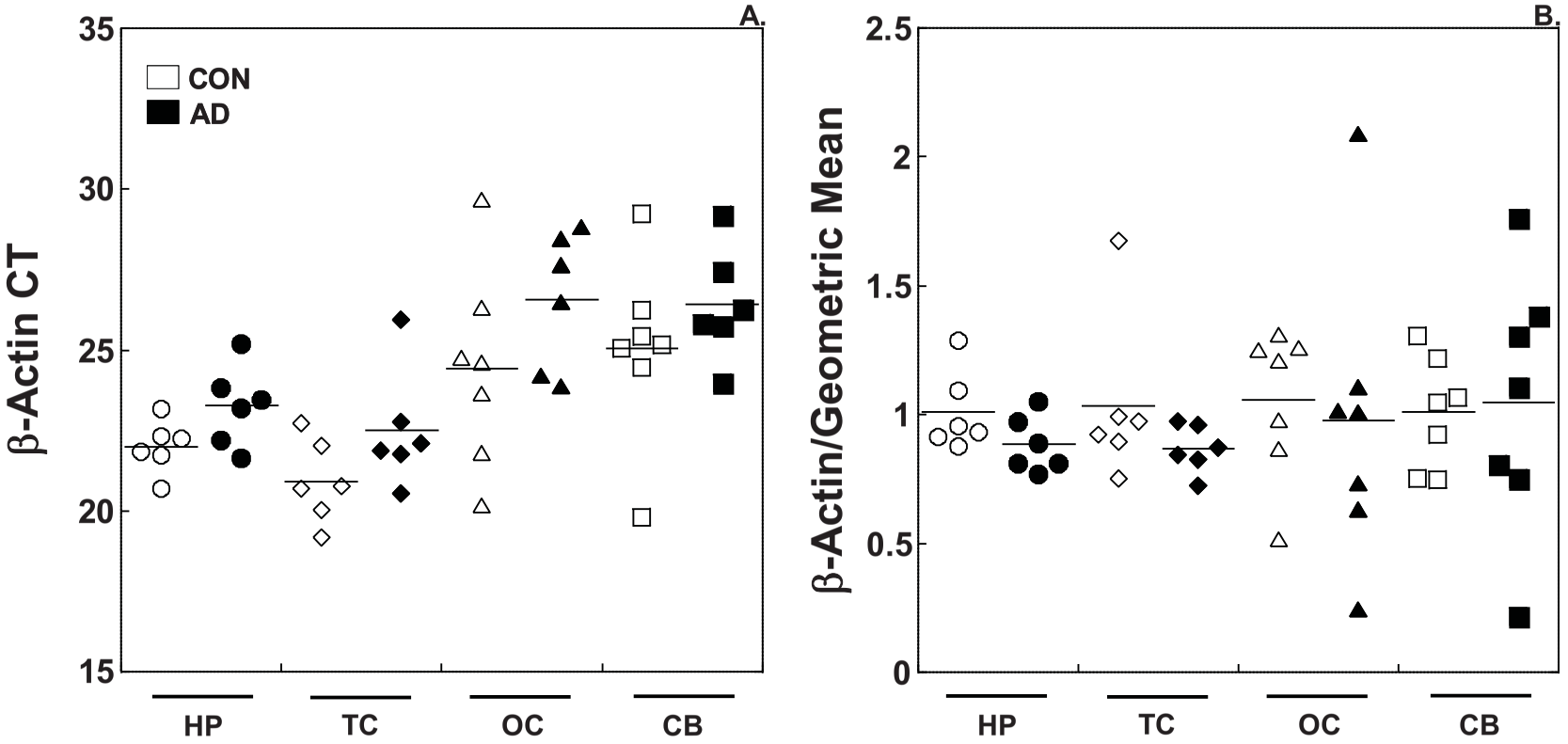

A.
CON
AD
β-Actin CT
35
30
25
20
15
HP
TC
OC
CB
B.
β-Actin/Geometric Mean
2.5
2
1.5
1
0.5
0
HP
TC
OC
CB

Fig. S2.

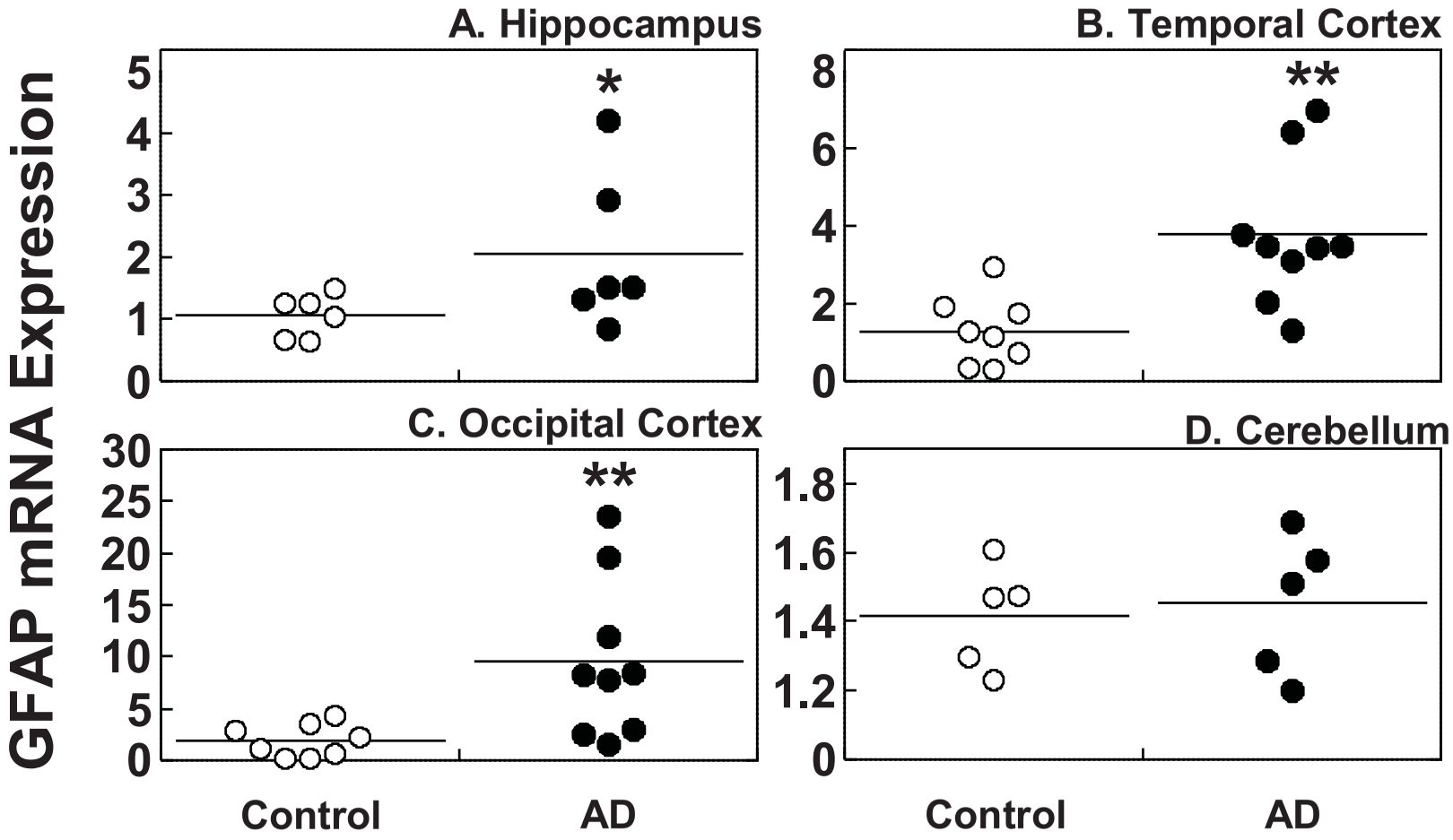

A. Hippocampus
B. Temporal Cortex
C. Occipital Cortex
D. Cerebellum
GFAP mRNA Expression
*
**
**
0
1
2
3
4
5
0
2
4
6
8
0
5
10
15
20
25
30
0
1.2
1.4
1.6
1.8
Control
AD
Control
AD

Fig. S3.